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DNA EXTRACTION FROM PRESERVED TROUT TISSUES

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Abstract.—We have adapted techniques developed for the extraction of DNA from formalin-fixed, paraffin-imbedded human tissues for use on preserved fish tissues. DNA was successfully extracted and the d-loop region of mitochondrial DNA was amplified with the polymerase chain reaction (PCR). The sequences of the amplified DNA from preserved and modern samples were identical. These techniques were also applied to fin tissue treated with a variety of preservatives. Extraction of DNA from ethyl alcohol and air-dried fin tissues gave yields equivalent to those from frozen tissues. Extraction of DNA from preserved museum specimens of rare or extinct taxa could significantly increase the scope of systematic and phylogenetic studies. Similarly, extraction of DNA from fin tissues provides a nonlethal sampling strategy allowing biochemical systematic analyses of rare or endangered taxa.

Key words: DNA sequencing, polymerase chain reaction, sequencing, cutthroat trout, Oncorhynchus.

As a part of our ongoing studies of the systematics of western salmonids, mainly cutthroat trout (*Oncorhynchus clarki*), we were interested in extracting DNA from preserved fish tissues. Museum collections contain many preserved specimens, usually stored in alcohol but originally fixed in formalin. These could represent a significant reserve of information for systematics research if the DNA could be successfully extracted. In addition, many populations of western trout are in such low numbers that collecting fish for systematic studies could seriously jeopardize their survival. For this reason we also wanted to evaluate the applicability of preserved-tissue DNA extraction techniques to samples of fin tissue. Fin samples could be taken rapidly in the field with minimal stress to the fish. These samples could then be preserved for later DNA extraction.

Medical researchers have developed techniques for the extraction of DNA from formalin-fixed, paraffin-imbedded tissues (Goetz et al. 1985, Debeau et al. 1986). The DNA extracted from these tissues was of sufficient quality that restriction cutting and southern blot analysis were possible (Debeau et al. 1986). DNA has also been successfully extracted from birds held in museum collections, both dried and preserved in 70% ethyl alcohol (Houde and Braun 1988). The DNA extracted from alcohol-preserved birds was significantly degraded (maximum size, 200 base pairs), while that from the dried tissues contained fragments 9–20 kb in length. But even if the DNA obtained with these procedures was degraded, the recent development of the polymerase chain reaction procedure (PCR) (Saiki et al. 1985, 1988, Mullis et al. 1986, Mullis and Faloona 1987, Wong et al. 1987, White et al. 1989) provides a technique to amplify specific fragments of DNA as small as 200 base pairs. These amplified fragments can then be sequenced to decipher genetic relationships (Saiki et al. 1985, Wrischnik et al. 1987, Kocher et al. 1989, Thomas and Beckenbach 1989).

Materials and Methods

Archived Specimens

Cutthroat trout collected between 1926 and 1982 and archived in the fish range at the Monte L. Bean Life Science Museum, Brigham Young University, were used to determine the usefulness of the formalin-extraction technique when applied to museum specimens. Samples of liver, muscle, or gut were taken from specimens representing a range of preservation times (Table 1). Tissues were removed from the specimens and placed in 20 volumes of TE9 buffer (300mM Tris, 20 mM EDTA, 10 mM NaCl, pH 9.0; Goetz

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TABLE 1. DNA yields from formalin-fixed museum specimens of cutthroat trout (*Oncorhynchus clarki*). DNA yields were determined using UV spectrometer absorbance readings at 260 nm.

Subspecies	Year	Location	Museum No.	Sample tissue type	Total weight (g)	DNA (μg)	DNA yield (μg/mg tissue)
O. c. bouvieri	1926	Snake R., ID	BYU #26792	liver	0.13	77.5	0.596
O. c. utah	1927	Utah L., UT	BYU #26755	liver	0.64	567.5	0.887
O. c. utah	1940	Utah L., UT	BYU #26756	liver	0.65	310.0	0.477
O. c. utah	1982	Deaf Smith, UT	BYU #176896	muscle	0.24	147.5	0.615
O. c. utah	1982	Deaf Smith, UT	BYU #176890	gut	0.42	965.0	2.298
O. c. utah	1928	Trout Cr., UT	BYU #26858	liver	0.07	51.0	0.728
O. c. utah	1981	Deep Cr., UT	BYU #176793	muscle	0.11	57.5	0.523

et al. 1985). The buffer was changed twice over 24 hours.

Fin Tissues

Fin tissues were taken from anesthetized hatchery rainbow trout (*Oncorhynchus mykiss*) that ranged in length from 15 to 25 cm. Samples were taken from all fins but were restricted to the outer edges of the fins to more accurately represent the region that would be sampled in the field. Approximately 1 cm^2 of fin was removed for each sample. These were placed in labeled 1.8-ml polyethylene tubes with gasketed screw caps. Four samples were taken for each of six treatments applied to the fins. These were (a) 10% formalin, (b) 40% isopropyl alcohol, (c) storage in a standard freezer at −20 C, (d) storage in an ultracold freezer set at −80 C, (e) 70% ethyl alcohol (EtOH), and (f) air-drying. The samples were held in the tubes for 45 days, after which the preservatives were decanted and the tissues soaked in TE9 for 24 hours, with no change in the buffer. The frozen and air-dried samples were not soaked in buffer prior to extraction. One sample stored at −20 C was lost during storage.

Extraction Procedure

Tissue samples were minced with a clean razor blade (to 2 mm or less in cross section) and placed in 15-ml centrifuge tubes with 10 ml of TE9 and 0.1 g of SDS. Five mg of proteinase K was added to each sample, and the tubes were capped and incubated in a shaking water bath for 24 hours at 55 C. An additional 5 mg of proteinase K and 0.1 mg SDS were added to each sample and the tubes returned to the shaking water bath for 50 hours at 55 C to remove residual undigested tissue. The samples were transferred to 30-ml tubes, and an equal volume of phenol-chloroform was added to each. The tubes were inverted several times to mix and then centrifuged in an SS-34 rotor at 10,000 rpm for 10 minutes. The aqueous phase from each sample was removed with an inverted glass pipette and placed into clean 30-ml tubes and the procedure repeated. A final extraction of the aqueous phase was made with one volume of chloroform and centrifuged as before. The aqueous phase from each sample was transferred to a new tube and .1 volume of 3 M sodium acetate solution added. The mixtures were precipitated with one volume of 95% EtOH and stored at −20 C overnight (12 hours minimum). Each sample was centrifuged at 10,000 rpm for 10 minutes and the supernatant carefully poured off, leaving a DNA pellet. The pellets were washed with 70% ethyl alcohol and centrifuged again for 10 minutes at 10,000 rpm. The alcohol was poured off and the samples allowed to air dry. The pellets were resuspended in a 3 mM Tris, 0.2 mM EDTA solution (pH 7.2). RNase was added to a final concentration of 20 μg/ml.

Results and Discussion

Archived Specimens

Muscle and liver tissues yielded comparable amounts of DNA, and exceptionally high yields were obtained from the sample of gut tissue (Table 1). Because the gut tissue was washed in buffer immediately after removal from the preserved specimen, contamination from items in the alimentary canal should have been minimal. Gut tissue was easily digested, indicating a relatively rapid release of DNA (Dubeau et al. 1986), and this could have been associated with the high yields. DNA samples (20 μl) from the museum specimens were electrophoresed on a

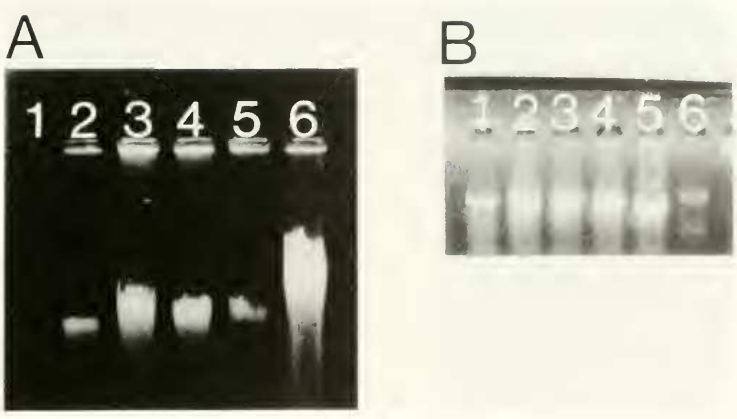

Fig. 1. DNA electrophoresed on 1% agarose gels after being extracted (Fig. 1A) from formalin-preserved museum specimens and following PCR amplification (Fig. 1B). The DNA from the trout collected in 1926 (liver) is only faintly visible (lane 1, Fig. 1A). The DNA from 1927 (liver), 1940 (liver), 1982 (muscle), and 1982 (gut) are in lanes 2–5, respectively. The DNA in lane 6 was extracted from a contemporary frozen liver sample. The PCR products are shown in Figure 1B. Lanes 1–6 in Figure 1B correspond to the DNA templates shown in lanes 1–6 in Figure 1A.

TABLE 2. A comparison of the nucleotide sequence (120 base pairs) from the SD-1 region of the mitochondrial DNA d-loop. The DNA was amplified with the polymerase chain reaction. The top row represents the base sequence from frozen-tissue DNA, and the lower row represents the sequence from a formalin-preserved specimen. The frozen-tissue specimen (BYU #90621) is *O. c. utah*, from McKinzie Creek, UT, collected 8-17-88. The preserved-tissue specimen (BYU #26755) is *O. c. utah*, from Utah L., UT, collected in 1927. Both vouchers are archived in the fish range at the Monte L. Bean Life Science Museum.

Frozen	A A G G C T A T C C	T T A A G A A A C C	A G C C C C T G A A	30
Preserved	A A G G C T A T C C	T T A A G A A A C C	A G C C C C T G A A	
	A G C C G A A G T A	A A G C A T C T G G	T T A A T G G T G T	60
	A G C C G A A G T A	A A G C A T C T G G	T T A A T G G T G T	
	C A A T C T T A T T	G C C C G T T A C C	C A C C A A G C C G	90
	C A A T C T T A T T	G C C C G T T A C C	C A C C A A G C C G	
	G G C T T C T C T T	A T A T G A C T A G	G G C C T C T C C C	120
	G G C T T C T C T T	A T A T G A C T A G	G G C C T C T C C C	

1% agarose gel containing ethidium bromide (Fig. 1A) to verify extraction. The DNA samples extracted from fresh and preserved tissue samples were used in a PCR reaction (25 µl total volume) using primers for the d-loop region of trout mitochondrial DNA developed by K. Thomas (University of California, Berkeley), with standard conditions (Perkin Elmer Cetus, Norwalk, Connecticut). Cycle times and temperatures were 1 minute at 92 C, 1 minute at 53 C, and 2 minutes at 72 C, for 35 cycles. PCR products are shown in Figure 1B. DNA extraction controls containing no fish tissue did not yield PCR products under identical conditions (data not shown). Subsamples of the PCR products from preserved and fresh tissue samples were sequenced (Fig. 2) and compared with contemporary sequence data from cutthroat trout (Table 2). The sequence data were identical, indicating that within the amplified segment no base modifications had occurred in the formalin-preserved sample.

Fin Clips

We obtained DNA from all fin clips regardless of preservation method. Mean yields ranged from a low of 0.40 µg/mg of tissue from formalin-preserved fin clips to a high of 1.104 µg/mg in air-dried samples (Table 3). The treatment effects were examined with analysis of variance (Table 4), and a highly significant difference was found between the treatments. Fisher's least significant difference multiple comparison procedure was applied to separate those treatment

Fig. 2 (at left). Sequence gel from a portion of the mitochondrial DNA d-loop. Column A is the sequence for a contemporary sample of trout DNA (BYU #90621) and column B is the sequence from a preserved trout specimen (BYU #26755) collected in 1927. The sequence gel is read from the bottom up, and the columns represent guanine (G), adenine (A), thymine (T), and cytosine (C), respectively.

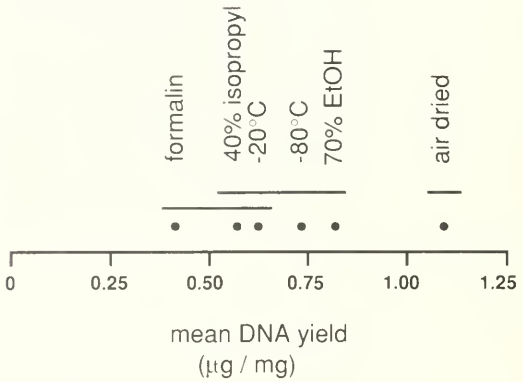

Fig. 3. Multiple comparisons of the means of the six fin tissue treatments, using Fisher's least significant difference test (alpha = 0.01). Lines connect means that do not differ significantly from one another.

TABLE 3. DNA yields from fin tissue preserved with different methods. The fin clips, approximately 1 cm^2 each, were taken from hatchery-reared rainbow trout (*Oncorhynchus mykiss*). DNA yields were determined using UV spectrometer absorbance readings at 260 nm.

Preservation method	N	Mean yield (µg/mg)	Standard deviation
formalin	4	0.402	0.15743
40% isopropyl	4	0.569	0.19111
−20 C	3	0.644	0.10016
−80 C	4	0.740	0.06295
70% EtOH	4	0.822	0.07964
air-dried	4	1.104	0.13443

groups that differed significantly from one another. These comparisons (Fig. 3) indicate that the air-dried treatment gave yields significantly higher than the other treatments. Because the weights used in calculating the DNA yields were the preextraction values and not the pretreatment weights, the initial weights (predrying) of the air-dried samples are not known. However, based on the initial size of the fin clips, they are assumed to have been similar. While air-drying yields are much better than

TABLE 4. One-way analysis of variance of the fin clip treatment effect on DNA yield

Source	Degrees of freedom	Sum of squares	Mean square	F	Prob > F
Treatment	5	1.14512	0.22902	13.47	0.0000
Error	17	0.28911	0.01700		
Total (adj)	22	1.43424			

those resulting from other preservation methods, the lack of preservatives could allow secondary contamination of samples through bacterial or fungal colonization, and air-drying probably should not be used in collecting samples in humid areas or where adequate storage is not possible. The yields obtained from ethyl alcohol preservation are equal to those from frozen tissues and superior to both isopropyl alcohol and formalin preservation. Of the preservatives examined in this study, ethyl alcohol would appear to be the preservative of choice in most field situations. This eliminates the necessity of carrying dry ice or liquid nitrogen into the field to preserve tissues. Other preservative solutions should be considered; for instance, Seutin, White, and Boag (1991) reported successful DNA extraction from avian tissues preserved in a mixture of EDTA, NaCl, and DMSO.

CONCLUSIONS

The ability to extract, amplify, and sequence DNA from formalin-preserved museum specimens increases the information value of museum holdings. In addition to being a record of morphological and meristic information, the specimens can be used in biochemical studies. Because museum collections include type specimens, rare species, and representatives of now extinct forms, many key phylogenetic relationships can be reexamined. The extraction techniques can be applied to contemporary preserved tissues as well. Fin tissues give adequate yields with this technique for both restriction enzyme digestion and PCR amplification. Fin samples, which can be taken nonlethally, present opportunities to examine fish populations that would otherwise be inaccessible to tissue collection because of management considerations.

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